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for Studying Neural Tube Induction of Skeletal Myogenesis

John C. Angello,[1] Howard M. Stern, and Stephen D. Hauschka

Department of Biochemistry, University of Washington, Box 357350, Seattle, Washington 98195

A model experimental system for investigating myogenic induction signals has been devised with mouse P19 embryonal carcinoma cells. When cocultured with pieces of chick neural tube, aggregated P19 cells are induced to become skeletal muscle. The most potent inducing activity is localized to the dorsal neural tube. Less activity was found in the ventral neural tube, notochord, ectoderm, and lateral plate mesoderm, and none was detected in the neural retina. These results suggest that P19 cells may be a useful model system for investigating the mechanisms underlying induction of somite myogenesis.

INTRODUCTION

Nascent somites consist of mesodermal cells which are not yet completely ''specified'' to a particular lineage (Aoyama and Asamoto, 1988; Ordahl and Le Douarin, 1992). Skeletal myogenesis in these cells depends on signals which emanate primarily from the axial tissues (neural tube and notochord) and also from the overlying ectoderm (Ordahl and Le Douarin, 1992; Rong *et al.*, 1992; Fan and Tessier-Lavigne, 1994; Buffinger and Stockdale, 1994, 1995; Münsterberg *et al.*, 1995; Münsterberg and Lassar, 1995; Stern *et al.*, 1995; Stern and Hauschka, 1995; Pownall *et al.*, 1996; Spence *et al.*, 1996). The biochemistry of the signaling process(es) remains largely unknown, although recent studies suggest that member(s) of the Wnt family (Münsterberg *et al.*, 1995; Stern *et al.*, 1995; Maroto *et al.*, 1997), sonic hedgehog (Münsterberg *et al.*, 1995; Maroto *et al.*, 1997), and/or combinations of TGF-β family members and bFGF (Stern *et al.*, 1997) may initiate inductions. Elucidation of these signal transduction pathways would benefit from an experimental system in which putative components could be readily manipulated. For this reason, we have explored the feasibility of using the pluripotent embryonal carcinoma cell line P19 as a signaling target. The P19 cell line and its responses to various inducing agents have been well-described (McBurney *et al.*, 1982; Edwards *et al.*, 1983). When treated with agents such as DMSO or retinoic acid, P19 cell aggregates can be induced to form skeletal myocytes, cardiocytes, neurons, and a variety of other terminally differentiated phenotypes. In this report, we show that P19 cells undergo skeletal myogenesis in response to signals from many of the same tissues which promote skeletal myogenesis in somites and that dorsal neural tube signals are especially effective, as they are in somite myogenic induction (Stern *et al.*, 1995).

[1] To whom correspondence should be addressed. E-mail: jca@u.washington.edu.

MATERIALS AND METHODS

Cells and culture. Mass cultures of P19 cells were grown in DMEM supplemented with 10% heat-denatured fetal bovine serum. Cells were routinely passaged before they reached confluence and were replated at 100K cells per 100-mm tissue culture dish, refed on day 2, and passaged again on day 3. For analysis of induction, P19 cell aggregates were formed as follows: mass cultures were collected by trypsin dissociation 1 day after passage (when the density was less than 5×10^5 cells per dish) and serially diluted. Thirty-microliter aliquots containing 50–100 cells were then plated on the underside of tissue culture dish lids (20 drops per 100-mm lid). An 8-ml reservoir of medium was placed in the dish to equilibrate the atmosphere around the drops, and cultures were maintained in a humidified 37°C, 5% CO_2 incubator. After 60 min, the cells had settled and could be found in a single microscope field, but they had not yet aggregated. At this time, the cells in representative drops were counted. When P19 cells were induced with DMSO, it was added at 0.6% (v/v) at the time of the final serial dilution, prior to ''plating'' the cells in hanging drops. When

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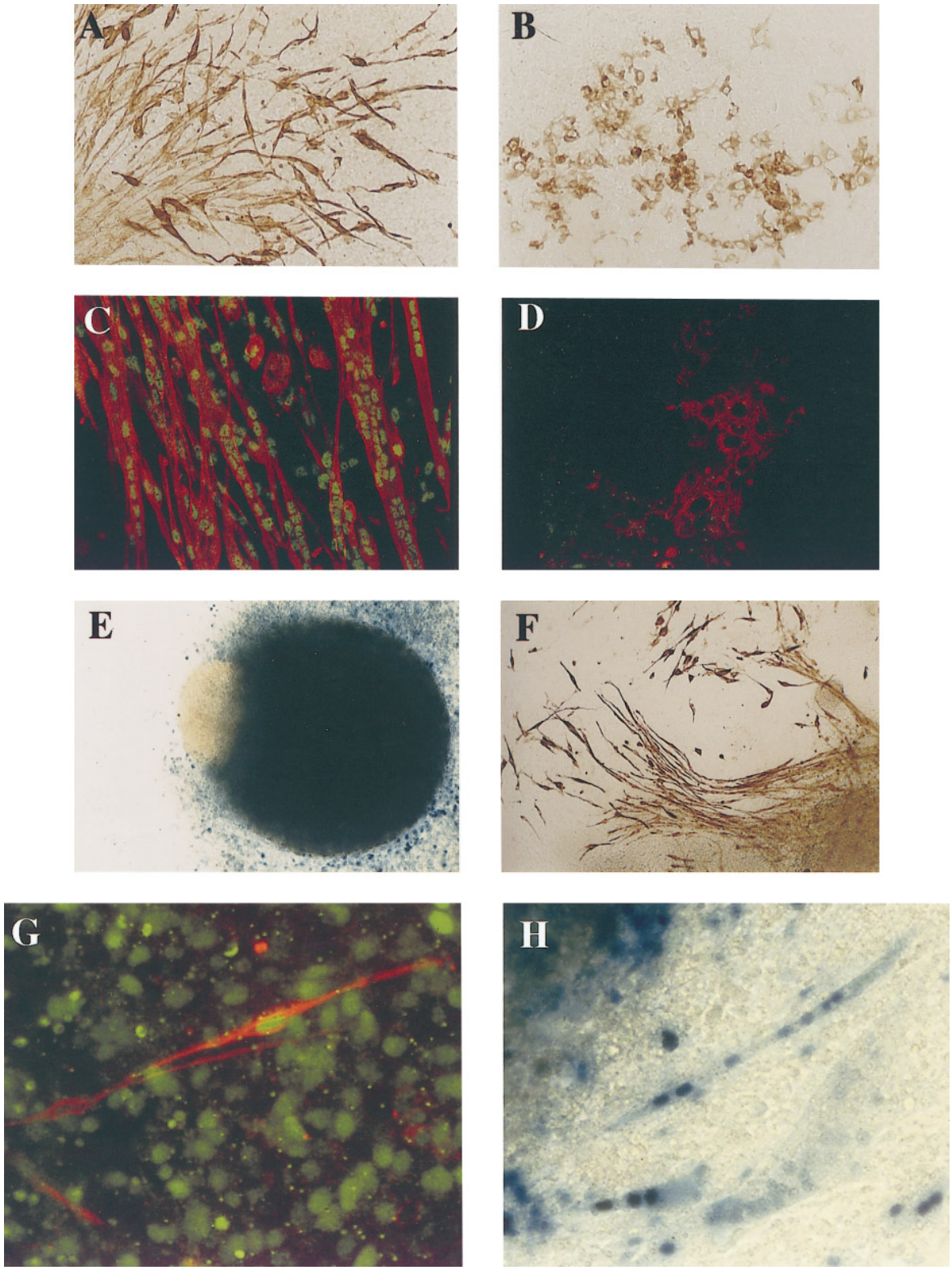

FIG. 1. Neural tube induces skeletal myogenesis in P19 cell aggregates. P19 cells, stably transfected with β-galactosidase (P19-nlacZ), were grown as hanging drop cultures for 5 days, transferred to an attachable surface for 2–10 days, and then assayed (see Materials and Methods). (A–D) Aggregates were formed in the presence of 0.6% DMSO. After 4 days of attachment and spreading, the resulting islands of cells were immunocytochemically stained for myosin heavy chain (A and B). The myosin-positive cells in A are skeletal muscle, and

pieces of neural tube or other tissues were cocultured with P19 cells, the undissociated pieces of tissue were added to the hanging drops in a 0.5- to 1-μl volume of medium within 4 h. On day 5, 5–10 P19 aggregates or P19/chick tissue coaggregates were transferred to separate locations within 100-mm plastic tissue culture dishes (an attachable surface) containing 10 ml of medium. Unless otherwise indicated, the resulting islands of cells were fixed and stained after 4 days. In most experiments, striated muscle was identified histochemically using MF-20, a monoclonal antibody specific to striated muscle myosin heavy chain (Bader *et al.,* 1982). In other experiments, the β-Gal activity in P19 cells stably transfected with constitutively expressed nlacZ was detected immunocytochemically with an X-gal solution (Pearson *et al.,* 1997). Alternatively, myosin and either β-Gal protein or myogenin were detected simultaneously with fluorescent secondary antibodies.

Transfection. A nuclear-targeted β-galactosidase gene under control of the cytomegalovirus promoter in a pUC plasmid (pUC-CMV-nlacZ) was generously provided by Mike Hauser and Jeff Chamberlain. P19 cells (10^6/100-mm plate) were transfected with 10 μg of this plasmid and 2 μg of a plasmid carrying a neomycin-resistance gene (Southern and Berg, 1982; Johnson *et al.,* 1992) using a standard calcium phosphate technique (Amacher *et al.,* 1993). Pools of stable transfectants were selected with 500 μg/ml geneticin and then cloned in order to obtain a highly expressing subclone.

Confocal microscopy. P19 cells induced with DMSO, and cocultures of P19 cells and pieces of neural tube, were assayed using confocal microscopy. The cultures were fixed with 100% MeOH for 20 min at −20°C and then stained simultaneously for myogenin and myosin heavy chain or for β-Gal protein and myosin heavy chain, respectively.

Dissections. Neural tubes, notochords, lateral plate mesoderm, and dorsal ectoderm were dissected from Stage 10–13 (E2) chick embryos, and neural retinas were dissected from E7 chick embryos (Stern and Hauschka, 1995; Lin-Jones and Hauschka, 1996). Neural tubes were dissected from between the rostral half of the segmental plate and somite VIII. They were then either cut transversely into 10 pieces of roughly equal size (designated ''whole'' neural tube) or first divided longitudinally into dorsal and ventral halves (see Stern *et al.,* 1995, Fig. 1, for example) which were then cut transversely into 10 pieces. Tissue other than neural tube was cut into pieces about the same size as the whole neural tube pieces.

RESULTS AND DISCUSSION

DMSO Induction of P19 Cells

In our experiments, we elected to form P19 cell aggregates in hanging drop culture for the following reasons: the initial cell number can be controlled and confirmed; the aggregation state of each culture can be readily assessed over time; individual aggregates and coaggregates can be assayed for induction; and the inductive responses can be more readily quantified. P19 hanging drop cultures begin to associate within 2 h and, by 24 h, form loose aggregates which thereafter evolve to more tightly associated spheroids. Whether or not DMSO is included in the medium, the population doubling time for the first 2–3 days is about 10 h, similar to that of cells attached to a plastic surface. After 5 days, P19 spheroids were individually transferred to 100-mm tissue culture plates, on which the spheroids attach and spread, forming islands of cells. Within 4 additional days, the aggregates are sufficiently spread so that individual cells can be distinguished. As determined by staining cultures fixed at this time with antibody to myosin heavy chain, striated muscle is *not* present when P19 cells are aggregated without DMSO. However, when this agent is included in the hanging drop cultures, myogenesis is induced (Table 1, lines 1 and 2). As described by others (McBurney *et al.,* 1982; Edwards *et al.,* 1983; Skerjanc *et al.,* 1994), skeletal muscle cells can be identified by their bipolar morphology (Fig. 1A) which clearly distinguishes them from cardiocytes, the other striated muscle cell type induced by DMSO (Fig. 1B). We have also confirmed the skeletal muscle phenotype of bipolar cells by staining for myogenin, a skeletal muscle-specific transcription factor (Figs. 1C and 1D).

Neural Tube Induction of P19 Cells

When a piece of neural tube is included in hanging drop cultures along with freshly dissociated nlacZ stably transfected P19 cells (to distinguish these from the chick embryo cells), the P19 cells initially form an apparent ''homoaggregate.'' By day 3, the P19 homoaggregate and the piece of neural tube begin to associate. When 5-day ''heteroaggregates'' are plated and then assayed for β-galactosidase activity 2 days later, it is clear that the vast majority of P19 and neural tube cells have remained self-associated. However, there are definite interfaces at which the P19 and neural tube cells are in direct contact and could

those in B are cardiocytes. After 10 days of attachment and spreading, islands were simultaneously stained with MF-20, a monoclonal mouse antibody to myosin, and a rabbit polyclonal anti-myogenin antibody. Myosin and myogenin were then localized with rhodamine- and fluorescein-conjugated secondary antibodies, respectively (C and D). C shows skeletal muscle cells which were incubated long enough to allow fusion. A few remaining bipolar, mononucleated cells can be seen. D contains images of cardiocytes, which are myosin-positive but myogenin-negative. (E) P19-nlacZ cells were cocultured with a piece of neural tube for 5 days, allowed to attach for 2 days, and then assayed for β-galactosidase activity. Note the areas of apparent close contact between the unstained neural tube tissue and the β-Gal-positive aggregated P19 cells. (F) A 5-day coculture of P19 cells and a piece of dorsal neural tube was allowed to attach and spread for 4 additional days and then was histochemically stained for myosin heavy chain. Note the many skeletal muscle cells. (G) Five-day cocultures of P19 cells and a piece of neural tube were allowed to attach and spread for 4 additional days and then were stained simultaneously with MF-20 and a rabbit polyclonal antibody to β-galactosidase. Myosin and β-galactosidase were localized with a rhodamine- and a fluorescein-conjugated secondary antibody, respectively. These cultures were assayed by confocal microscopy to ensure that myosin-positive skeletal muscle cells were truly derived from P19 precursors. (H) Similar cocultures were allowed to attach and spread for 10 days and then were assayed for β-galactosidase activity. Multinucleated, β-galactosidase-positive cells confirm that skeletal muscle was derived from P19 precursors.

TABLE 1
Induction of Skeletal Muscle in P19 Cell Aggregates by Different Embryonic Tissues

Cells/tissues	*n*	*n′*	% with skeletal muscle
1. P19 cells alone	6	130	0
2. P19 + DMSO	6	106	95
3. P19 + neural tube	4	83	66
4. P19 + notochord	3	75	9
5. P19 + ectoderm	3	73	21
6. P19 + lateral plate mesoderm	3	61	16
7. P19 + neural retina	3	68	0

Note. n, number of separate experiments. *n′,* total number of islands scored. P19 cell aggregates were formed in hanging drop cultures either alone, under ''inducing conditions'' (i.e., in the presence of 0.6% DMSO), or in coculture with pieces of embryonic tissue (see Materials and Methods). After 5 days, cultures were transferred onto 100-mm plates for attachment and assayed 4 days later for the presence of myosin-positive, skeletal muscle cells. In representative coculture experiments with P19 cell aggregates and pieces of *neural tube,* the number of skeletal muscle cells per positive coculture was 40.4 ± 6.4 (SE) (27 cocultures; range, 2–111 myosin positive cells). On average, fewer myosin-positive cells were found in positive cocultures of P19 cell aggregates with the other inducing tissues (see text).

potentially interact (Fig. 1E). Four days after plating such cocultures, differentiated skeletal muscle cells can be found within the islands (Fig. 1F and Table 1, line 3). On average, the number of skeletal muscle cells is 40.4 ± 6.4 (SE). When pieces of neural tube are cultured alone under these same conditions, *no* myosin-positive cells are found, showing that no chick myoblasts contaminate the dissected neural tube tissue. To unequivocally demonstrate that the skeletal muscle obtained from cocultures is generated from P19 cells, cultures were stained for both β-galactosidase protein and myosin heavy chain and then were analyzed by confocal microscopy. An image of a β-Gal(+)/myosin(+) cell is illustrated in Fig. 1G and demonstrates that the neural tube can induce P19 cells to express the skeletal muscle phenotype. Consistent with this observation, multinucleated cells with β-galactosidase-positive nuclei are identified in longer-term cocultures (Fig. 1H).

Relative Inductive Capacities of Dorsal and Ventral Neural Tube

Ablation experiments (Spence *et al.,* 1996) and *in vitro* reconstitution experiments (Fan and Tessier-Lavigne, 1994; Stern *et al.,* 1995) have shown that the *dorsal* neural tube is a much more effective promoter of somite myogenesis than is the ventral neural tube. A similar result was found when skeletal myogenesis was induced in P19 cells by the dorsal and ventral neural tube (Table 2). Furthermore, when multiple pieces of dorsal neural tube were cocultured with P19 cell aggregates, skeletal muscle was induced nearly 100% of the time. It is not yet established whether this increased activity is due solely to more aggregated P19 cells being in close contact with neural tube cells (refer to Fig. 1E) or whether soluble factors are involved.

Similar to previous results with somite cultures (Buffinger and Stockdale, 1995; Stern *et al.,* 1995), ventral neural tube pieces also induce myogenesis in P19 cocultures; however, the response of P19 cell aggregates to a single piece of ventral neural tube is weak and several pieces fail to increase the myogenic response (Table 2).

Induction of P19 Cells by Tissues Other Than Neural Tube

Under the appropriate assay conditions, notochord and dorsal ectoderm also promote somite myogenesis (Kenny-Mobbs and Thorogood, 1987; Fan and Tessier-Lavigne, 1994; Stern and Hauschka, 1995; Cossu *et al.,* 1996; Pownall *et al.,* 1996; Spence *et al.,* 1996). Likewise, these tissues induce P19 cells to become skeletal muscle, although, as expected, the apparent inducing potentials are less than that of the neural tube: less than one-third as many notochord and dorsal ectoderm cocultures exhibited P19 skeletal myocytes (Table 1, lines 4 and 5). Furthermore, the number of induced skeletal muscle cells per positive coculture (8.7 ± 6.7 and 27.1 ± 5.9, respectively) is less than that obtained with the neural tube (see above).

Lateral plate mesoderm (LPM) also was found to be a weak inducer of skeletal muscle in P19 cells (Table 1, line 6; and 28.5± 11.8 skeletal muscle cells per positive coculture), comparable to that of dorsal ectoderm. This was not anticipated because LPM inhibits myogenesis in migrating limb muscle precursor cells (Pourquié *et al.,* 1995, 1996), possibly due to its production of the TGF-β family member BMP-4. In addition, Buffinger and Stockdale (1994), Stern and Hauschka (1995), and Cossu *et al.* (1996) have reported that LPM does not promote somitic myogenesis *in vitro.* At this time, we cannot account for these apparent discrepancies. However, LPM is not a homogeneous tissue (Hayashi and Ozawa, 1995), and the hanging drop coculture conditions

TABLE 2
The *dorsal* Neural Tube Contains Most of the P19 Cell-Inducing Activity

Cells/Tissues	*n*	*n′*	% with skeletal muscle
P19 cells alone	3	60	0
P19 + dorsal NT (1 piece)	3	36	64
P19 + ventral NT (1 piece)	3	37	14
P19 + dorsal NT (3–5 pieces)	2	15	93
P19 + ventral NT (3–5 pieces)	2	16	13

Note. n, number of separate experiments. *n′,* total number of islands scored. See Materials and Methods and legend to Table 1.

may permit expression of LPM factors supportive of myogenesis, at least in P19 cells.

In contrast to the results with axial tissues, LPM, and ectoderm, neural retina is *inactive* as an inducer of skeletal myogenesis both in somites (Stern and Hauschka, 1995) and in P19 aggregates (Table 1, line 7). It is not known whether this is due to a lack of myogenic inducing factors, per se, in neural retina or to the simultaneous presence of inhibitors which could mask inducing activity.

An early step in somite myogenesis presumably involves the expression of myogenic determination factors (MDFs) (Weintraub *et al.*, 1989). MDF transcription in cells of the dorsomedial somite presages the emergence of the myotome and the epaxial muscles and is thought to occur in response to neural tube/notochord signals (Münsterberg and Lassar, 1995; Pownall *et al.*, 1996). Both in mouse (Ott *et al.*, 1991; Cossu *et al.*, 1996) and in chick (Maroto *et al.*, 1997), low levels of myf-5 transcription precede a more robust expression of MyoD, which is apparently promoted by neural tube factors (Bober *et al.*, 1994; Pownall *et al.*, 1996). Similarly, P19 cells do not produce MyoD—even when the cells are aggregated—unless an inducer is present (Skerjanc *et al.*, 1994). Furthermore, Skerjanc *et al.* (1994) have shown that, upon their aggregation, MyoD-transfected P19 cells can express a skeletal muscle phenotype. These results, and our data, suggest that, as with somite cells, MDF transcription may be a key process underlying neural tube induction of P19 cell aggregates. Since P19 cells can be readily transfected, it should be possible to create P19 sublines which will be useful in testing this hypothesis and dissecting the inductive mechanism(s) of skeletal myogenesis.

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